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Anticancer properties of nanoparticle synthesized from Cyphostemma auriculatum Roxb. on nude mice

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***Abstract*---**The present study was aimed to establish the pharmacological and therapeutic properties of a green synthesized silver nanoparticles (AgNPs) in breast cancer induced by 7,12-dimethylbenzanthracene (DMBA) in nude mice. In this study, AgNPs made from *Cyphostemma auriculatum* Roxb. leaf extract(CA-AgNPs) were tested in a nude mice model for anticancer activity. A significant elevate changes in blood chemistry like heamoglobin, RBC, WBC, platelets and also on blood biochemical parameters such as catalase and SOD with obtained after 28 days of treatment with carcinogen. However, these levels were restored to normal at the end of the study period treated with CA-AgNPs. The liver oxidative stress enzymes showed no significant alterations. With 15 and 30 mg/kg b.w of CA-AgNP, histopathological analysis revealed no significant abnormalities in the kidney, spleen, lungs, heart, testis, or brain. However, 30 mg/kg b.w. of CA-AgNPs caused considerable cell edema and vacuolar degeneration in the liver, which returned to normal at the conclusion

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of the washout period. The findings of this study suggest that green produced CA-AgNPs at low concentrations could be beneficial.

***Keywords*---**Anticancer, *Cyphostemma auriculatum* Roxb., DMBA, CA-AgNPs.

Introduction

Nanotechnology has experienced phenomenal growth in recent years as a result of its numerous applications in sectors such as material science, chemistry, medicine, nanobiotechnology, and others. Nanoparticles (NPs) have attracted a lot of attention because of their high surface-to-volume ratio and extremely small size, which leads to differences in physical and chemical properties when compared to bulk materials with the same composition [1]. Due to several improved approaches created by researchers for the synthesis of NPs with defined size and form to fulfill highly particular criteria, there has been significant progress in the nanotechnology research field in the recent times. New applications for NPs are fast emerging, and numerous patents and research papers describing new nanoparticle synthesis techniques are published each year. The weight unit of a specific material in the Nano size scale has a much higher surface area than the same weight unit for the same material in the macro scale size due to its incredibly small size. Particle composition, morphology, crystallinity, size, and shape all influence the intrinsic properties of metal nanoparticles.

Silver NPs (AgNPs) have piqued researchers' interest among the NPs due to their wide variety of applications in antibacterial, catalysis, medicinal, optical, and energy (2-14). Intensive study has been done based on these findings to investigate their qualities and possible applications for a variety of objectives, including antimicrobial agents in wound dressings, anticancer agents, electronic devices, and water purification. *In-vivo* investigations of AgNPs made using diverse chemical methods revealed toxicity. The size, concentration, coating, and distribution level of AgNPs have an impact on their behaviour. Alternatively, to synthesis NPs without using harsh and expensive toxic chemicals, microbial and biological system (green chemistry) techniques are being developed. Because of their environmentally favourable creation of NPs, microorganism-based NPs synthesis is quickly developing. However, the microbial technique has drawbacks because it takes more effort to maintain colonies. Because of their natural availability, quick creation, and environmentally beneficial character, NPs are being produced using various plant sources.

Cancer is a category of diseases that cause a variety of pathogenic and metabolic alterations in cells. Cell proliferation, angiogenesis, and metastasis are all examples of signaling processes that contribute to its development (15-18). Aerobic glycolysis, mitochondrial DNA depletion, and changes in respiratory chains and genetic expressions are all aberrant metabolic processes in cancer cells. The second biggest cause of death in women is breast cancer. The high prevalence of breast cancer has had a significant influence on society. The majority of breast cancers are estrogen-dependent, and 30-40% of patients who

receive adjuvant tamoxifen medication experience a relapse (19). As a result, endocrine treatment resistance appears to be a clinical issue. Cancer treatments, both physical and pharmacological, are limited at various stages. Currently existing medicines, on the other hand, have a negative impact on normal cell functioning while exposing patients to excessive drug and radiation exposures.

In earlier reports AgNPs were synthesized using *Cyphostemma auriculatum* Roxb., leaf extract. *Cyphostemma auriculatum* Roxb., is used as a folklore medicine and blood purifier in cardiac disorders, intestinal worm diseases, earache, wound abscess, dog bite, rheumatism, purulent wounds, wound healing, tumors, cough, colds, and hydrocele, but also as a tonic and an astringent, according to an ethnomedicinal survey (20). This herb has also been used to treat animal bloody dysentery and diarrhea in veterinary medicine. Alkaloids, flavonoids, saponins, steroids, terpenoids, stilbenoids, and tannins have been found in Vitaceae family plants, according to phytochemical investigations. In a previous paper, described the synthesis and characterization of silver nanoparticles (21). The AgNPs were tested *invivo* in this study to see how effective they are against breast cancer. DMBA (7,12-dimethylbenz(a)anthracene), a prototype of polyaromatic hydrocarbons (PAH), induced breast cancer model on nude mice for *invivo* evaluations among the different preclinical rodent models investigated for breast cancer studies.

Materials and Methods

Sigma-Aldrich, Merck, and Himedia provided all organic compounds and solvents required for all assays and biological studies. The rest of the compounds were of analytical grade.

In-vivo studies

Acclimatization of animals

Thirty six female nude mice (20 ± 2 g) were purchased from National institute of nutrition (Hyderabad, India). All animals were kept in clean, sanitized PVC coated stainless steel cages in an air-conditioned animal house with typical climatic conditions such as a constant temperature of 20–25°C, relative humidity of 45–55%, and a 12 hour light–dark cycle. Animals were provided a regular pellet meal and free access to water before and throughout the experiment. Animals were fasted for 12 hours before administering experimental treatments, but were allowed proper access to water. All of the trials were carried out during the day. Before the experiment, the animals were given a 7-day acclimatization period.

Preparation of DMBA and experimental design for treatment-oriented study

Sigma Chemicals in Mumbai, India, provided the DMBA, which was stored at 20°C to avoid decomposition and dissolved in olive oil carrier. The typical medication, tamoxifen citrate, was dissolved in ethanol (100 mg/mL) and dilute it hundred fold in distilled water to get a final concentration of 1mg/mL. The plant crude and CA-AgNPs also dissolved in ethanol and dilute in distilled water. The mice were placed into six groups, each with six rodents. Animals with tumors were chosen to receive therapy for 28 days. Except for the control group, all 30

animals were given a single gastric intubation in 1 ml olive oil to induce mammary carcinogen (25 mg/kg b.w), during which the Latency period (the number of days between the DMBA injection and the emergence of the first tumor in each mice) was recorded. Palpation revealed the presence of a tumor. Animals with tumors were chosen for treatment for 28 days.

Experimental design

The mice were split into six groups, each with six mice (n = six). The trial lasted a total of 28 days. All of the mice in the study were fed a standard pellet diet. The experiment was conducted in six groups of six mice each as mentioned below.
Group-1: Normal saline treated mice (Normal control-NC)
Group-2: Cancer induced Nude mice (given DMBA (25 mg/kg) by single gastric intubation in 1 mL olive oil.
Group-3: Cancer induced Nude mice (given DMBA (25 mg/kg) by single gastric intubation in 1 mL olive oil + crude plant extract 120 mg/kg body weight
Group-4: Cancer induced Nude mice (given DMBA (25 mg/kg) by single gastric intubation in 1 mL olive oil + AgNPs 15 mg/kg body weight
Group-5: Cancer induced Nude mice (given DMBA (25 mg/kg) by single gastric intubation in 1 mL olive oil + AgNPs 30 mg/kg body weight.
Group-6: Cancer induced Nude mice (given DMBA (25 mg/kg) by single gastric intubation in 1 mL olive oil + tamoxifen citrate 10 mg/kg body weight

Blood samples were collected on Day-01, Day-07, Day-14, Day-21, and Day-28. The animals were sedated on the 28th day, blood was obtained through the retro-orbital sinus and utilized to study hematological parameters, and the blood was centrifuged and the serum was collected. Biochemical parameters were investigated using the serum obtained. After the blood was collected, the animals were decapitated and the entire liver and kidney were perfused with ice-cold 0.9 percent sodium chloride. The organs were then carefully removed, trimmed free of superfluous tissue, and the mammary tumors were excised and histological effects evaluated.

Histopathology

Tissues having gross pathological alterations, as well as normal tissue, were cut into thin slices of 3 to 5 mm thickness. Keep the tissue in a 10% formalin fixative at room temperature for 24-48 hours. Xylol was used to deparaffinize the portion for 5 to 10 minutes before pure alcohol was used to remove the xylol. After cleaning the segment, it was stained with hematoxylin for 3-4 minutes before being counterstained with 0.5 percent eosin for 15 to 30 seconds until the section became bright pink. Blotted and dehydrated in alcohol for 15 to 30 seconds before clearing with xylol. Keep the slide dry and free of air bubbles by mounting it on a Canada balsam or DPX mutant.

Determination of serum biochemical parameters

Hemoglobin content, WBC, RBC, and platelet counts were all measured in the blood samples. Various biochemical parameters such as SOD and catalase were measured in the serum collected after centrifugation of the collected blood. The

activity of superoxide dismutase (SOD) was determined by adding 0.5 mL of serum sample to 1.5 mL of carbonate buffer pH10.2, 0.5 mL of 0.1 Mm EDTA, and 0.4 mL of epinephrine to 1.5 mL of carbonate buffer pH10.2, and reading the OD at 480 nm. SOD activity was measured in units/min/mg protein. The amount of enzyme that decreases the rate of adrenaline autoxidation by 50% is defined as one unit of the enzyme. Catalase activity was determined spectrophotometrically by the method of Koroliuk et.al (22). Briefly 0.1mL of sample was incubated with 100 µmol/mL of H_2O_2 in 0.05 mmol/L Tris-HCl buffer (pH-7) for 10 minutes. The reaction was terminated by rapidly adding 0.5 mL of 4% ammonium molybdate. Yellow complex of ammonium molybdate and H_2O_2 was measured at 410nm. One unit of catalase activity was defined as the amount of enzyme required to decompose 1µmol H_2O_2/minute.

Results and Discussion

The presence of a miniscule amount of silver is usually non-hazardous (23). However, the toxicity of AgNPs has remained a source of debate due to the potential for harm when utilised in biological systems. For further successful use in future applications in biology and medicine, it is required to examine the *in vivo* effect of green synthesized AgNPs in animal models.

Histopathology of breast tissue

The segment of breast derived from normal mice showed no signs of cancer in the histology reports (Fig. 1a). Infiltrating neoplasm constituted of cells grouped in glands was found in tumor-induced mice with only DMBA treatment, as seen in the image. Individual cells were round to oval in shape, with somewhat eosinophilic cytoplasm and round oval vesicular nuclei, with nucleoli visible in some (Fig.1b). Neoplastic cell mass slightly replaced in plant crude treated mice (Fig.1c). AgNP-treated, DMBA-induced mice tissue slices from the breast exhibited a confined lesion with diffused hyperplasia of ducts (adenosis) separated by fibro collagenous stroma at low doses (15 mg/kg body weight). A cystic lesion constituted of disseminated neutrophil infiltration was discovered in DMBA mice sections treated with a high dose of CA-AgNPs (30 mg/kg body weight). Neoplastic cell mass replaced more than plant crude treated mice in CA-AgNPs treated mice (Fig.1d & Fig.1e). Tamoxifen-treated DMBA-induced mice breast tissue revealed a circumscribed lesion with areas of adenosis and fibrosis and mostly neoplastic mass are replaced with fibrous tissues. There was no evidence of malignancy (Fig. 1f). Congested arteries and fibro-collagenous tissue were visible in the stroma. There was no evidence of malignancy. The mice given 15 mg/kg b.w of CA-AgNPs had normal hepatic parenchyma, but the mice given 30 mg/kg b.w of CA-AgNPs had histological changes such as significant cell swelling and vacuolar degeneration. These histological changes, however, were not seen in mice following the washout period. The kidney, spleen, lungs, heart, testis, and brain did not show any histopathological changes after being treated with 15 and 30 mg/kg b.w of CA-AgNPs.

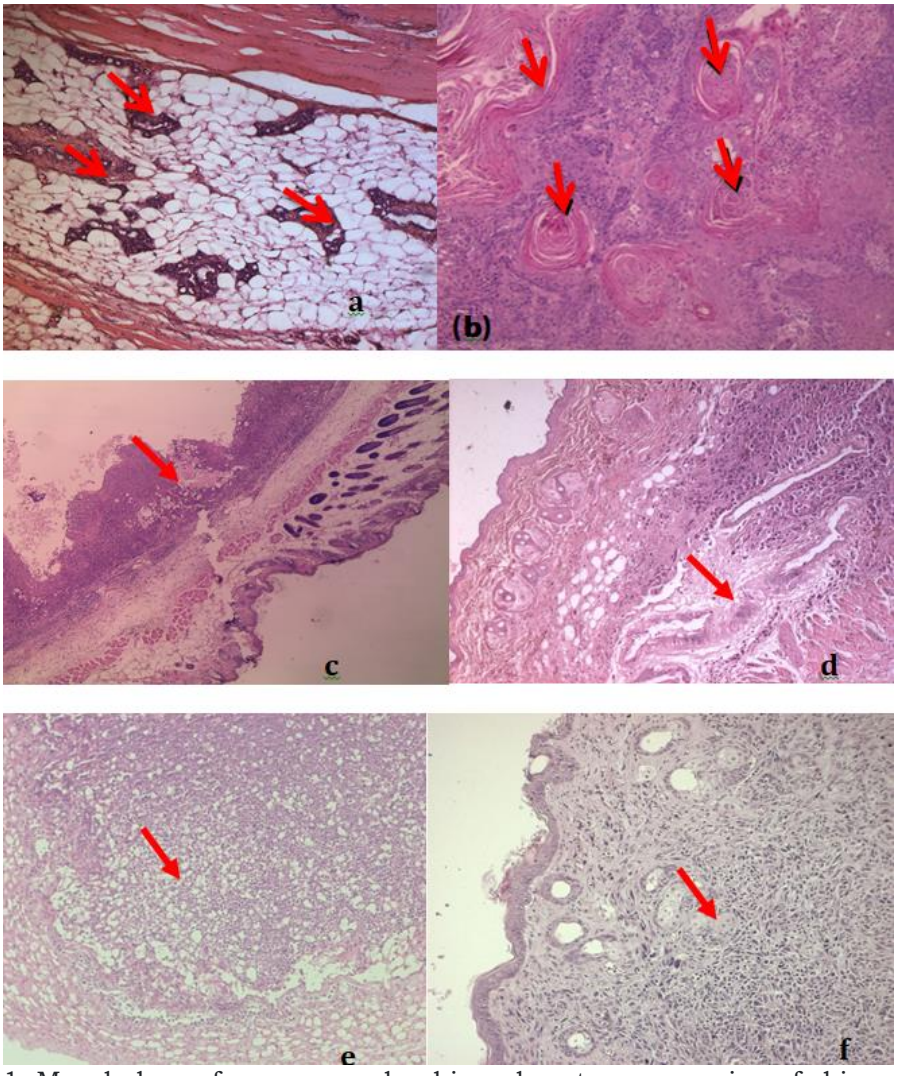

Figure 1: Morphology of mammary gland in sub-cutaneous region of skin
(1a) Morphology of mammary gland in normal control mice (Group-I) (x100). (1b) Large area of squamous metaplasia noticed in between tumor mass (Group-II) (x200). (1c) Neoplastic cell mass slightly replaced with fibrous tissue (Group-III) (x400). (1d & 1e) Neoplastic cell mass replaced with fibrous tissues in CA-AgNPs treated mice (Group- IV & V)(x 400 and x500), (1f) Neoplastic mass mostly replaced with fibrous tissues in tamoxifen treated mice (Group- VI)(x500).

Biochemical markers

Because oxidative stress is linked to cancer, antioxidant experiments were conducted *in vivo* on DMBA-induced mammary carcinoma in mice models. The enzymes listed below were investigated. In the event of cancer, the transport function of cell organelles, particularly hepatocytes, is disrupted, resulting in the release of enzymes and increased plasma membrane permeability. In tumor-induced mice, recent studies have found an increase in tumor volume. At various

time intervals and washout periods, anti-oxidant enzymes such as SOD and CAT were examined. In CA-AgNPs treated mice, serum antioxidant levels increased significantly in a time-dependent manner as compared to control group mice, but there was significant difference between the 15 and 30 mg/kg b.w treated group mice. In comparison to control mice, CA-AgNPs treated mice showed less significant changes in other biochemical parameters such as haemoglobin, RBC, WBC, and platelets, as well as anti-oxidant enzymes like SOD and catalase.

Effect of AgNPs on Catalase

When DMBA-induced mice were compared to normal mice, the catalase level was found to be 51.6 unit/mg lower. It appears that the decline is statistically significant. Catalase levels rose when DMBA-induced mice were given *Cyphostemma auriculatum* Roxb.,leaf extract (120 mg/kg b.w) and low and high dose of (15 and 30 mg/kg b.w) CA-AgNPs. When low and high dose (15 and 30 mg/kg b.w) CA-AgNPs were given, catalase levels increased in a concentration and time dependent manner and nearly equal to the results observed for standard drug Tamoxifen-treated DMBA mice. (Figure-2).

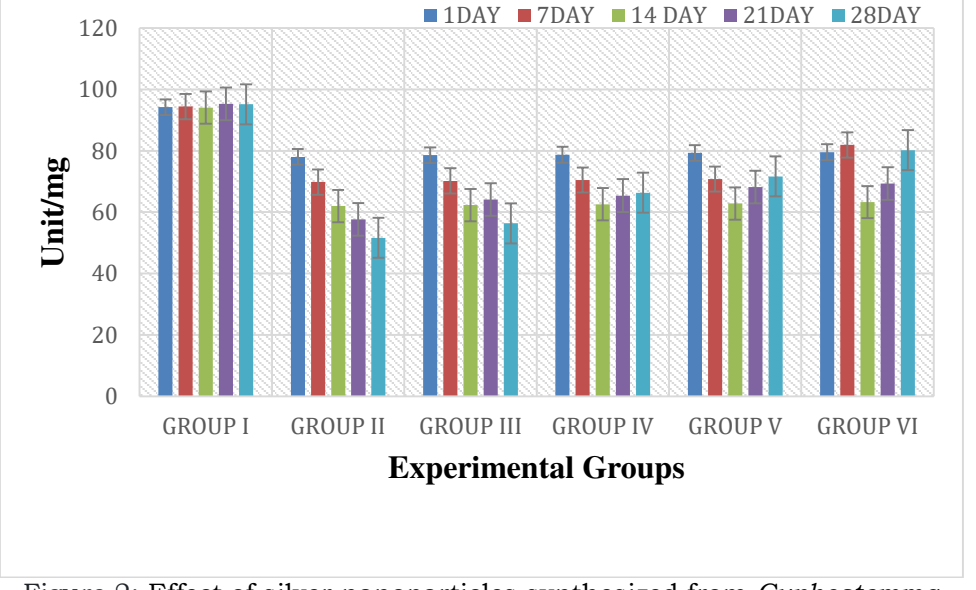

Figure 2: Effect of silver nanoparticles synthesized from *Cyphostemma auriculatum* Roxb. on Catalase

Effect of AgNPs on Superoxide dismutase

DMBA-induced mice had a 3.25 unit/mg lower SOD level than normal mice. It appears that the drop is statistically significant. SOD levels rose in DMBA-induced mice treated with *Cyphostemma auriculatum* leaf extract (120 mg/kg) and low and high dose of (15 and 30 mg/kg b.w) CA-AgNPs. When AgNPs of low and high doses (15 and 30 mg/kg b.w) were given, SOD levels increased in a concentration and time dependent manner, as seen in Fig.3 and nearly equal to the results observed for standard drug Tamoxifen-treated DMBA mice.

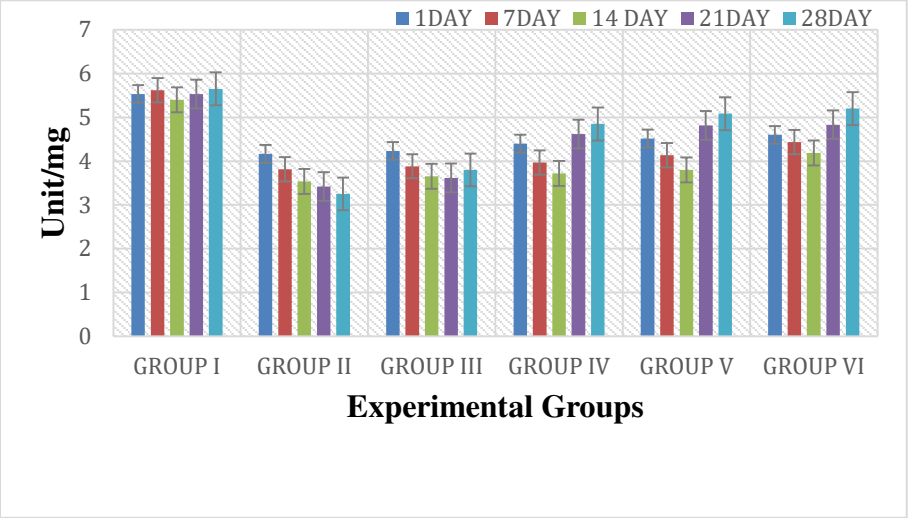

Figure 3: Effect of silver nanoparticles synthesized from *Cyphostemma auriculatum* Roxb. on SOD

In our study, group II (DMBA-induced mice) showed a significant decrease in antioxidant enzyme levels compared to group I (normal). Conversely, group III (plant extract 120 mg/kg treated mice), group IV (CA-AgNPs 15 mg/kg treated mice) and group V (CA-AgNPs 30 mg/kg), group VI (tamoxifen-treated DMBA-induced mice), showed a significant increase in the level of antioxidant enzymes levels compared to group II.

Hematological parameters

Glycation of proteins such as haemoglobin occurs when blood glucose levels are high (Gupta et al., 1997), resulting in the generation of reactive oxygen species (ROS), which leads to increased lipid peroxidation and cytotoxicity (Anwer et al., 2007). Hematological parameters of cancer patients are often substantially lower than usual. In the current investigation, a significant decrease in the Hemoglobin, RBC, and platelets level and a significant increase WBC level was observed in DMBA-induced mice, compared to normal mice. In CA-AgNPs treated DMBA-induced mice, an increase in Hemoglobin, RBC, and platelets levels were observed compared to DMBA-induced mice (Group-II). Significantly in CA-AgNPs treated DMBA-induced mice, a decrease in WBC levels compared to DMBA-induced mice (Group-II) were observed. The protective effect of CA-AgNPs is supported by these findings. By measuring blood Hemoglobin, RBC, WBC and platelets level, the impact of CA-AgNPs was determined. The results seem to be statistically significant. When DMBA-induced mice were treated with *Cyphostemma auriculatum* Roxb. leaf extract (120 mg/kg), the hemoglobin, RBC, and platelets levels increased and WBC levels decreased. When CA-AgNPs of low and high dose (15 and 30 mg/kg b.w) were administered a concentration and time dependent increase in Hemoglobin, RBC, and platelets levels and a decrease in WBC levels were observed as seen in Fig. 4,5,6 and 7.

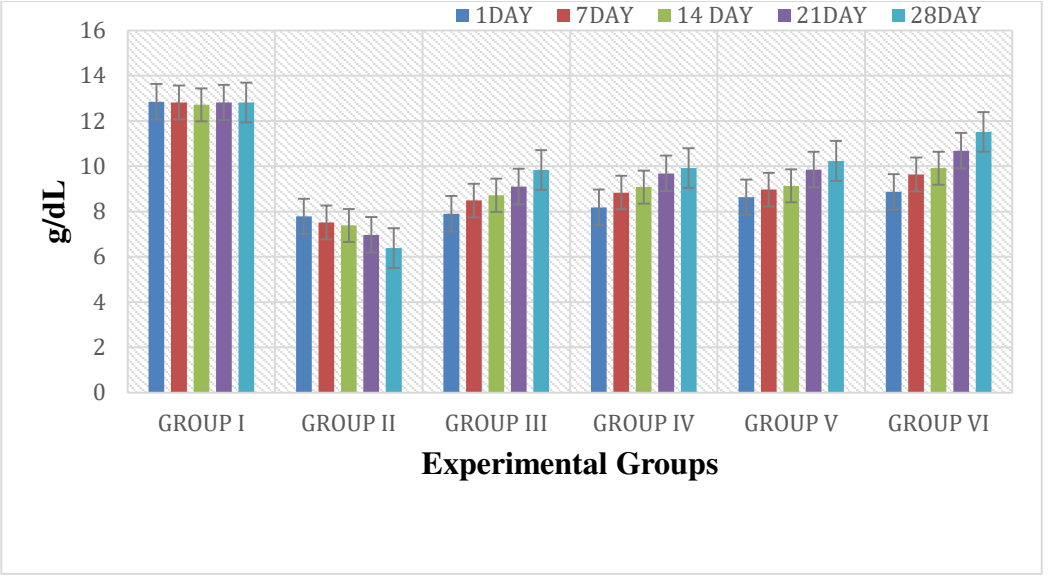

Figure 4: Effect of silver nanoparticles synthesized from *Cyphostemma auriculatum* Roxb. on Hemoglobin

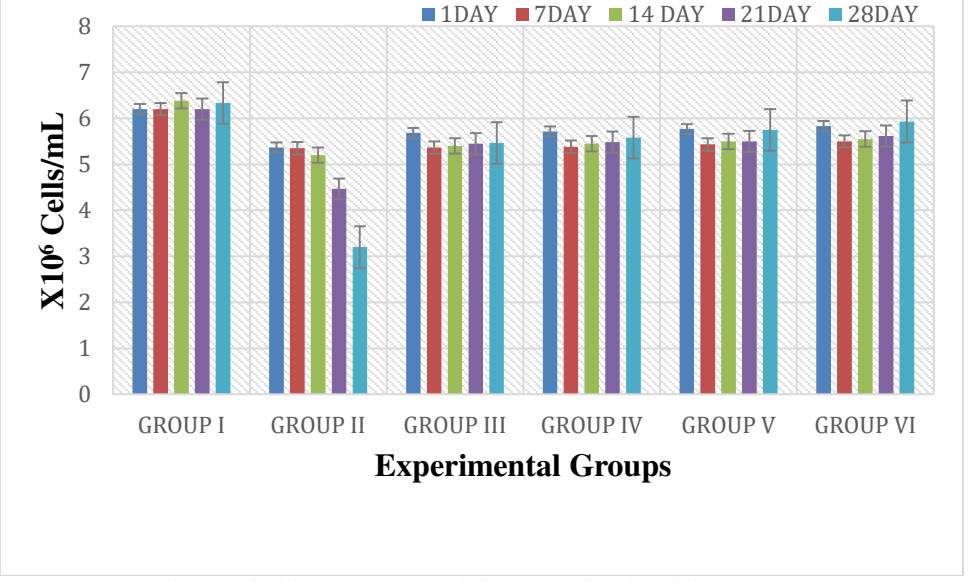

Figure 5: Effect of silver nanoparticles synthesized from *Cyphostemma auriculatum* Roxb. on RBC

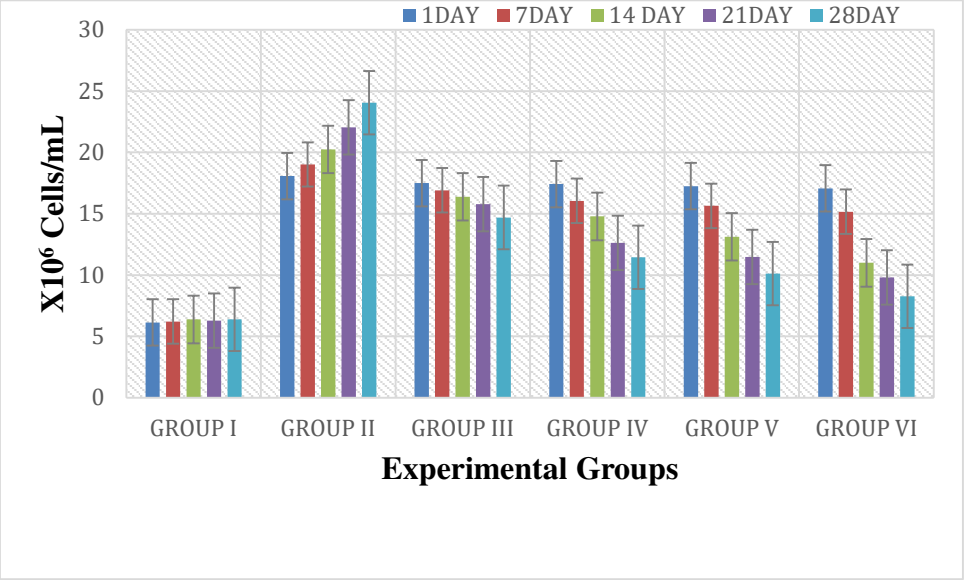

Figure 6: Effect of silver nanoparticles synthesized from *Cyphostemma auriculatum* Roxb. on WBC

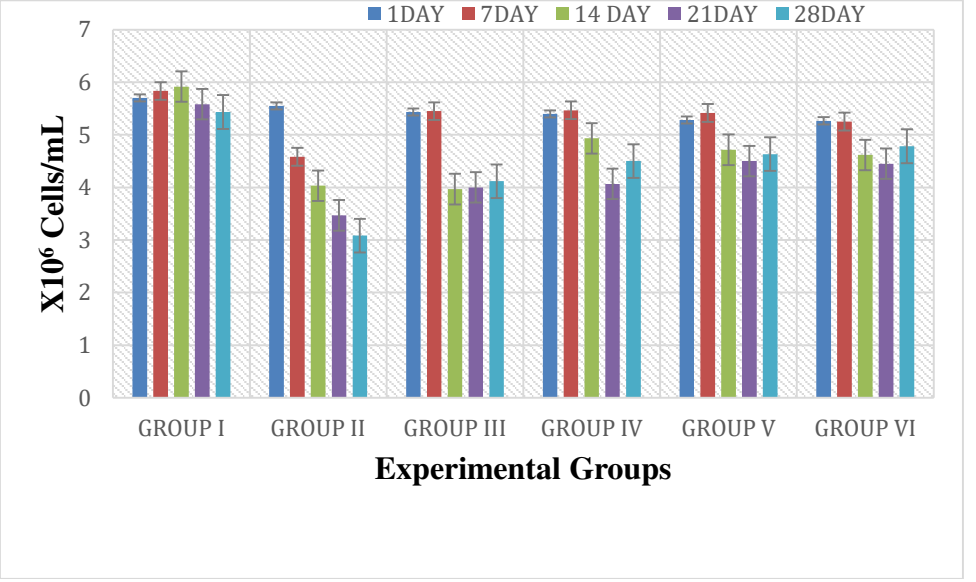

Figure 7: Effect of silver nanoparticles synthesized from *Cyphostemma auriculatum* Roxb. on Platelets

Conclusion

Finally, we were able to gain a better knowledge of the possible anticancer properties of green produced CA-AgNPs. The findings of our *in vivo* investigation confirm our belief that, if further investigated, this could lead to the development

of a novel therapeutic agent with medically and economically significant implications for saving the lives of troubled and oppressed people.

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Conflict of Interest

The authors declare no conflict of interest.

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